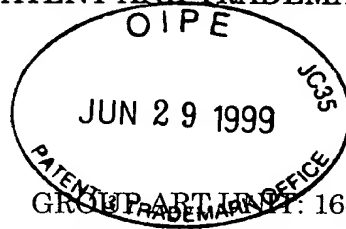


IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



APPLICANTS: Motomi NAKATA et al.

SERIAL NO: 08/913,555

FILED: September 19, 1997

EXAMINER: DAVID SAUNDERS

FOR: MONOCLONAL ANTIBODY SPECIFICALLY REACTING WITH Fas LIGAND  
AND PRODUCTION PROCESS THEREOF

DECLARATION UNDER 37 C.F.R. § 1.132

I, Motomi NAKATA, a citizen of Japan residing at c/o Osaka Works of Sumitomo Electric Industries, Ltd., 1-33, Shimaya 1-chome, Konohana-ku, Osaka-shi, Osaka 554-0024 Japan, do declare and say as follows:

1. I graduated from the Faculty of Engineering, the Department of Fermentation Technology, Osaka University, in March 1984, and finished the Master's Course of said Department in March 1986.

2. Since April 1986, I have been employed by Sumitomo Electric Industries, Limited, an assignee of the above identified application, and engaged in research in the field of bioengineering and immunology at the Research Laboratory of said company.

3. My bibliography is attached as Appendix A.

4. I have read and understand the specification and the pending claims in the above-identified patent application.

5. I have read and understand the rejection under 35 U.S.C. § 112, first paragraph, asserted in the final Office Action, dated April 1, 1999. In essence, the final Office Action asserts that the specification did not enable one of skill in the art at the time of the invention to prepare the Fas/WR19L cell line recited in claim 54 without use of a deposit of the biological material.

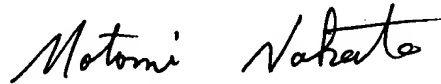
6. One of skill in the art at the time of the invention could produce the Fas/WR19L cell line without reference to a deposit of the biological material using the method referenced at page 51, lines 17-26, of the present specification.

7. One of skill in the art could use the repeatable method disclosed in Hanabuchi et al., Proc. Nat. Acad. Sci. USA, Vol. 91, No. 11, pp. 4930-4934, disclosed at page 51 of the

present specification and attached hereto, to produce the Fas/WR19L cell line recited in claim 54. WR19L cells are available from ATCC (ATCC TIB52). The transfection of the human Fas gene into WR19L can be prepared by reference to Hanabuchi et al. Page 4931, left hand column, of Hanabuchi et al. discloses a method for the "Preparation of Mouse Fas Transfectants." One of skill in the art could obtain the Fas/WR19L cell line in accordance with the procedures disclosed therein by employing human Fas gene and WR19L in place of mouse Fas gene and L5178Y cells, respectively.

8. I further declare under penalty of perjury pursuant to the laws of the United States of America that the foregoing statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date; June 18, 1999



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Motomi Nakata

Attachments:

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Hanabuchi et al. reference.

## APPENDIX A

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Proc. Natl. Acad. Sci. USA  
Vol. 91, pp. 4930-4934, May 1994  
Immunology

## Fas and its ligand in a general mechanism of T-cell-mediated cytotoxicity

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Communicated by Leonard A. Herzenberg, February 14, 1994

**ABSTRACT** To investigate the mechanisms of T-cell-mediated cytotoxicity, we estimated the involvement of apoptosis-inducing Fas molecule on the target cells and its ligand on the effector cells. When redirected by ConA or anti-CD3 monoclonal antibody, a CD4<sup>+</sup> T-cell clone, BK1, could lyse the target cells expressing wild-type Fas molecule but not those expressing death signaling-deficient mutants. This indicates the involvement of Fas-mediated signal transduction in the target cell lysis by BK1. Anti-CD3-activated but not resting BK1 expressed Fas ligand as detected by binding of a soluble Fas-Ig fusion protein, and the BK1-mediated cytotoxicity was blocked by the addition of Fas-Ig, implicating the inducible Fas ligand in the BK1 cytotoxicity. Ability to exert the Fas-mediated cytotoxicity was not confined to BK1, but splenic CD4<sup>+</sup> T cells and, to a lesser extent, CD8<sup>+</sup> T cells could also exert the Fas-dependent target cell lysis. This indicates that the Fas-mediated target cell lysis pathway can be generally involved in the T-cell-mediated cytotoxicity. Interestingly, CD4<sup>+</sup> T cells prepared from *gld/gld* mice did not mediate the Fas-mediated cytotoxicity, indicating defective expression of functional Fas ligand in *gld* mice.

Cytotoxic T lymphocyte (CTL)-mediated cytotoxicity constitutes an important component of specific effector mechanisms in immunosurveillance against virus-infected or transformed cells, but the molecular mechanisms have not yet been fully understood. So far, two models for lymphocyte-mediated cytotoxicity have been proposed—the granule exocytosis model and the induced suicide model (1–4). Recently, the effector molecules in the former, such as perforin (4) and granzymes (5), have been identified and it has been confirmed that the perforin-dependent mechanism constitutes one primary pathway of CTL-mediated cytotoxicity by the transfection (6) and gene targeting (Hidefumi Kojima and N.S., unpublished data; ref. 44) studies. However, accumulating evidence has demonstrated that the perforin-dependent mechanism is not the sole pathway for CTL-mediated cytotoxicity. It has been reported that certain T-cell clones could lyse target cells without an apparent expression of perforin (7). Moreover, genetically perforin-deficient T cells have also been found to exert cytotoxicity against certain target cells (Hidefumi Kojima and N.S., unpublished data; ref. 44). We also have reported that several class II-restricted soluble antigen-specific murine CD4<sup>+</sup> T-cell clones, including BK1 (used in the present study), could efficiently lyse the cognate antigen-presenting cells without a detectable level of perforin (8). The target cell lysis exerted by these clones was associated with target cell DNA fragmentation (one of the characteristics of apoptotic cell death) and was contact-

dependent and TNF-independent. This meets the induced suicide model, but the molecules on effector and target cells responsible for this type of killing remain unclear.

Recently, Rouvier *et al.* (9) demonstrated the involvement of target cell surface Fas molecule in the cytotoxicity exerted by certain CTLs. The Fas (alternatively termed APO-1) molecule was initially identified by monoclonal antibodies (mAbs) that induce apoptotic cell death upon binding to certain tumor cells (10, 11). Molecular cloning of the Fas antigen (12) revealed that it belongs to the emerging family of cell surface receptors including tumor necrosis factor receptors (13–15), CD40 (16), CD27 (17), CD30 (18), and 4-1BB (19), the specific ligands for which are all presented on the cell surface as type II integral membrane proteins (20–23). Moreover, very recent cloning of the Fas ligand revealed that it is also a type II integral membrane protein homologous to tumor necrosis factor (24). Taken together, this knowledge strongly implies that target cell death potentially mediated by the intercellular interaction between Fas on the targets and its ligand on effectors may be fully responsible for the induced suicide model. Although Rouvier *et al.* (9) have demonstrated the requirement of Fas expression on the target cell surface for being lysed by certain CTLs, they did not indicate the involvement of death signal transduction through the target cell surface Fas. In the present study, we first addressed this issue by using the target cells expressing signaling-deficient mutant Fas. More important, we also characterized the expression and function of the Fas ligand on T cells by utilizing the Fas-dependent target cell lysis system and a soluble Fas-human IgG Fc fusion protein (Fas-Ig).

### MATERIALS AND METHODS

Cells. BK1 is a keyhole limpet hemocyanin (KLH)-specific I-E<sup>d</sup>-restricted CD4<sup>+</sup> T-cell clone derived from BALB/c mice. It was maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (J.R. Scientific, Woodland, CA) and 5% culture supernatant of ConA-stimulated rat spleen cells and stimulated every 2 weeks with KLH and irradiated BALB/c spleen cells as described (8). A20HL was derived from a BALB/c B lymphoma, A20.2J [American Type Culture Collection (ATCC)], by transfection with anti-2,4,6-trinitrophenyl (TNP) IgM genes and was used as cognate antigen-presenting cells for BK1 after sensitization with TNP-KLH (8). A20HL and a DBA/2 T lymphoma, L5178Y (ATCC), were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Thymocytes were pre-

Abbreviations: CTL, cytotoxic T lymphocyte; E/T, effector to target; Fas-Ig, mouse Fas-human IgG1 Fc; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; NP, nitrophenol; PE, phycoerythrin; KLH, keyhole limpet hemocyanin.

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pared from 5-week-old MRL+/+ and MRL *lpr/lpr* mice (The Jackson Laboratory) or CBA+/+ and CBA *lpr<sup>g</sup>/lpr<sup>g</sup>* mice (Institute of Medical Science, University of Tokyo). Splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells were prepared from nylon wool-nonadherent BALB/c, C3H+/+, or C3H *gld/gld* (Sankyo) spleen cells by depletion of the opposite subset and residual B cells with mAbs (anti-CD8.3.155; anti-CD4.RL172.4; anti-B cell.J11d) plus complement as described (25). In some cases, BK1 clones were stimulated on culture dishes coated with 10 µg of anti-CD3 mAb per ml for 3–24 hr.

**Antibodies.** A hamster anti-mouse CD3 mAb (145-2C11) (26) was purified from ascites by using mAb TrapG (Pharmacia) affinity chromatography. Fluorescein isothiocyanate (FITC)-labeled goat anti-rat IgG, FITC-labeled goat anti-hamster IgG, and phycoerythrin (PE)-labeled goat anti-human IgG antibodies were purchased from Caltag (South San Francisco, CA). A heteroconjugated antibody (2C11αNP) was prepared by chemically conjugating 145-2C11 and an anti-nitrophenol (NP) hapten mAb with *N*-succinimidyl-3-(2-pyridyldithiol)propionate (Pharmacia) as described (27). Hamster anti-mouse Fas mAbs (P4-4 and RK-8) and rat anti-mouse Fas mAbs (RMP6 and RMP9) were raised against recombinant soluble mouse Fas molecule and identified by their reactivity with the mouse Fas transfectants described below (Y.N. and S.Y., unpublished data).

**Cytotoxicity Test.** Cytotoxicity tests were performed in V-bottomed wells of 96-well microtiter plates with <sup>51</sup>Cr-labeled target cells (10<sup>4</sup> tumor cells or 10<sup>5</sup> thymocytes) and effector cells at the indicated effector-to-target (E/T) ratios in the presence of ConA (10 µg/ml) or 2C11αNP (0.5 µg/ml) for NP-modified target cells. NP modification of the target cells was carried out with NP-*O*-succinimide as described (28). After 4–16 hr of incubation, aliquots (100 µl) of the supernatants were collected and counted on a γ-counter. Percent specific <sup>51</sup>Cr release was calculated as described (28).

**Preparation of Mouse Fas Transfectants.** The cDNA encoding the truncated mouse Fas molecule (90 amino acids had been removed from the C-terminal end) was obtained by PCR using oligonucleotides corresponding to nucleotides 27–46 and 740–760 of the published sequence (29). LS178Y cells were cotransfected with *Eco*RI-digested pMAMneo (Clontech) and *Pvu*I-digested pME18S (30) containing mouse Fas cDNA encoding whole molecule (mFas) or truncated mutant Fas (mFasΔCyt) by electroporation at 290 V with a capacitance of 960 µF using a Gene Pulser (Bio-Rad). A-1 transfectant expressing normal Fas (mFas/LS178Y) and F-10 transfectant expressing truncated Fas (mFasΔCyt/LS178Y) were obtained after G418 (0.4 mg/ml) selection and cloning by limiting dilution.

**Construction of Fas-Ig.** The cDNA encoding the leader sequence and extracellular portion of the mouse Fas molecule was obtained by PCR using TCACTCGAGATGCTGTGGATCTGGGCTGT as the 5' primer and TCAGGATCCGATTTCTGGGACTTTGTTT as the 3' primer. A *Xho*I site and a *Bam*HI site were introduced into the 5' and 3' primers, respectively. After *Xho*I and *Bam*HI digestion, the PCR product of the expected size was isolated from the agarose gel and subcloned into *Xho*I- and *Bam*HI-digested pBluescript II SK(+) (Stratagene). After confirmation of the sequence on an automated fluorescent DNA sequencer (Applied Biosystems), the 510-bp *Xho*I-*Bam*HI fragment containing the extracellular region of mouse Fas cDNA and the 1.3-kb *Bam*HI-*Xba*I fragment containing human IgG1 constant region genomic sequences, which was prepared from an IgG1 expression plasmid (31) provided by Brian Seed (Massachusetts General Hospital, Boston), were subcloned into *Xho*I- and *Xba*I-digested pBluescript II SK(+). This results in the in-frame fusion of the mouse Fas extracellular region to the human IgG1 hinge region (Fas-Ig). After digestion with *Xho*I and *Nor*I, a 1.8-kb insert containing the fusion

construct was transferred into the *Xho*I and *Nor*I sites of the CDM8 expression vector (31) also provided by Brian Seed. Production of the Fas-Ig fusion protein by transient expression in Cos cells and purification by protein A-Sepharose (Pharmacia) affinity chromatography were performed as described (31). SDS/PAGE analysis of the purified Fas-Ig protein indicated that it was produced as a disulfide-linked homodimer, and enzyme-linked immunosorbent assay (ELISA) indicated that it reacted with all anti-mouse Fas mAbs tested (data not shown).

**Flow Cytometry Analysis.** To examine the reactivity of Fas-Ig against BK1, 1 × 10<sup>6</sup> cells were incubated with 1 µg of purified Fas-Ig for 1 hr at 4°C followed by PE-labeled anti-human IgG. After washing with phosphate-buffered saline, the cells were analyzed on a FACScan (Becton Dickinson) and data were processed by using the Consort 30 program.

## RESULTS AND DISCUSSION

**BK1 Exerts Fas-Mediated Target Cell Lysis.** A murine CD4<sup>+</sup> T-cell clone, BK1, efficiently lysed the cognate antigen-presenting cell A20HL without an apparent expression of perforin (8). The target cell death exerted by BK1 was accompanied by apoptotic DNA fragmentation (8). Our initial preliminary observation that A20HL highly expressed Fas and was highly susceptible to an agonistic anti-mouse Fas mAb (RK-8) suggested the possible involvement of Fas in the BK1-mediated cytotoxicity (data not shown). To test whether BK1 can exert target cell lysis through target cell surface Fas, we first used mouse Fas transfectants as the target cells. Mouse T lymphoma LS178Y cells, which did not express Fas as estimated by Northern blotting and immunofluorescent staining with anti-mouse Fas mAbs (data not shown), were introduced with mouse Fas cDNA encoding the entire coding region to make mFas/LS178Y. We also introduced a mutant Fas whose cytoplasmic region required for death signaling was deleted to make mFasΔCyt/LS178Y. After G418 selection, we obtained mFas/LS178Y (clone A-1) and mFasΔCyt/LS178Y (clone F-10), which expressed Fas on their surfaces to similar levels as estimated by immunofluorescent staining with anti-mouse Fas mAbs (data not shown). As expected, LS178Y, which did not express Fas, and the mFasΔCyt/LS178Y, which expressed death signaling-deficient Fas, were totally resistant to an agonistic anti-mouse Fas mAb, but the mFas/LS178Y was highly susceptible (data not shown). Since LS178Y did not express class II and could not present the specific antigen (KLH) for BK1, we tested the cytotoxic activity of BK1 against these target cells in the presence of ConA (Fig. 1A Left) or in the presence of heteroconjugated anti-CD3 and anti-NP hapten antibody (2C11αNP) against NP-modified target cells (Fig. 1A Right). In either condition, only mFas/LS178Y was lysed by BK1, whereas both LS178Y and mFasΔCyt/LS178Y were totally resistant. This indicates that the BK1-mediated cytotoxicity requires not only the expression of Fas molecule on the target cells but also the death signal transduction through the target cell surface Fas.

The involvement of Fas-mediated signal transduction in the BK1 cytotoxicity was also tested by using *lpr* and *lpr<sup>g</sup>* thymocytes as the target cells. As reported by Ogasawara et al. (32), MRL+/+ thymocytes but not MRL *lpr/lpr* thymocytes expressed Fas on the surface as estimated by immunofluorescent staining with anti-mouse Fas mAbs (data not shown). This defect has been shown to result from insertion of the retrotransposon in the *lpr* Fas gene (33). In contrast, CBA+/+ thymocytes and CBA *lpr<sup>g</sup>/lpr<sup>g</sup>* thymocytes expressed Fas at equivalent levels but only the former was susceptible to an agonistic anti-Fas mAb (data not shown), as reported for MRL *lpr<sup>g</sup>/lpr<sup>g</sup>* thymocytes (32). This signaling

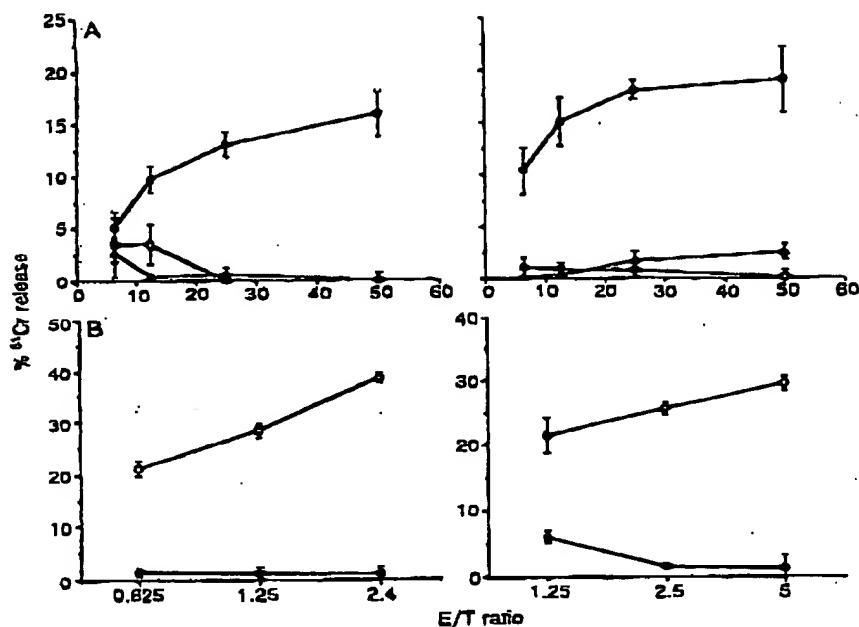


FIG. 1. Fas-mediated cytotoxicity exerted by BK1. (A) BK1 cytotoxicity was tested against LS178Y (○), mFas/LS178Y (●), and mFasΔCyt/LS178Y (▲) in the presence of 10  $\mu$ g of ConA per ml (Left) or 0.5  $\mu$ g of 2C11xNP per ml (Right) in a 6-hr <sup>51</sup>Cr release assay. Data represent mean  $\pm$  SD of triplicate samples. No cytotoxicity was detected in the absence of ConA or 2C11xNP (data not shown). (B) BK1 cytotoxicity was tested against MRL+/+ (○) or lpr/lpr (●) thymocytes (Left) and CBA+/+ (○) or lpr<sup>ce</sup>/lpr<sup>ce</sup> (●) thymocytes (Right) in the presence of 10  $\mu$ g of ConA per ml in a 6-hr <sup>51</sup>Cr release assay. Data represent mean  $\pm$  SD of triplicate samples. No cytotoxicity was detected in the absence of ConA (data not shown).

defect has been shown to result from a point mutation in the cytoplasmic region of the *lpr*<sup>ce</sup> Fas (34). In the presence of ConA, BK1 lysed the MRL+/+ and CBA+/+ thymocytes but neither of those from MRL *lpr/lpr* or CBA *lpr*<sup>ce</sup>/*lpr*<sup>ce</sup>. This further implicates the Fas-mediated signal transduction in the target cell lysis by BK1.

**Expression of Fas Ligand on BK1.** The involvement of target cell surface Fas in the BK1-mediated cytotoxicity suggested that BK1 might express the Fas ligand, which triggers the target cell lysis upon binding to the target cell surface Fas. The above observations that BK1 lysed mFas/LS178Y only in the presence of ConA or 2C11xNP suggested that the Fas ligand was not constitutively expressed on BK1 but might be induced by T-cell receptor (TCR)/CD3-mediated stimulation. Inconsistent with this supposition, BK1 efficiently lysed mFas/LS178Y in the absence of ConA when preactivated with immobilized anti-CD3 mAb (data not shown). To directly characterize the expression of putative Fas ligand, we constructed a soluble fusion protein composed of the extracellular portion of mouse Fas and the Fc portion of human IgG (Fas-Ig). Similarly composed fusion proteins have been successfully used to identify the specific ligands for CD40 (20), CD27 (21), CD30 (22), and 4-1BB antigen (23),

all of which belong to the same family of membrane receptors as Fas (12–19). A substantial binding of the Fas-Ig to BK1 was detected only after anti-CD3 stimulation (Fig. 2), indicating that the Fas ligand is inducible upon engagement of the TCR/CD3. Although the molecular nature of the Fas ligand on BK1 detected by Fas-Ig remains to be determined, Suda et al. (24) recently performed expression cloning of the Fas ligand by using a similarly composed Fas-Ig and identified a 40-kDa type II integral membrane molecule that is homologous to tumor necrosis factor and directly cytotoxic to the cells expressing Fas.

**Involvement of Fas Ligand in BK1 Cytotoxicity.** We then tested whether the Fas-Ig blocked the BK1 cytotoxicity against mFas/LS178Y (Fig. 3). The Fas-Ig, but not the control human IgG, did inhibit the BK1 cytotoxicity in a dose-dependent manner. This clearly indicates the functional

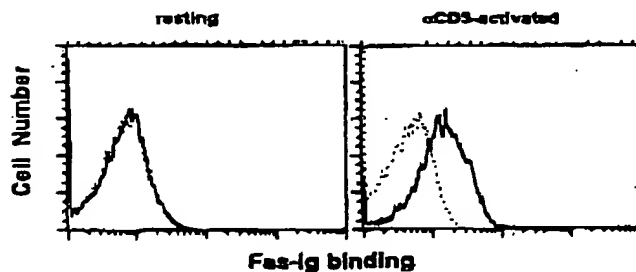


FIG. 2. Fas-Ig binding to anti-CD3-activated BK1 cells. BK1 cells, either resting (Left) or activated with immobilized anti-CD3 mAb for 3 hr (Right), were incubated with 1  $\mu$ g of Fas-Ig followed by PE-labeled anti-human IgG (solid lines). Dotted lines indicate background staining with the second-step antibody alone.

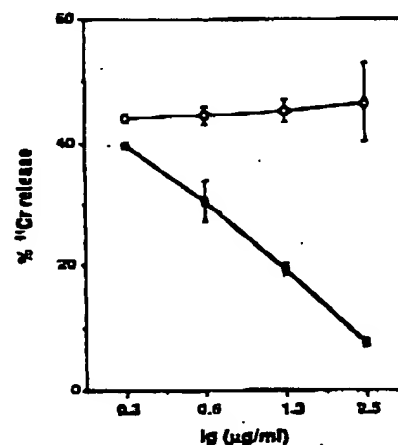


FIG. 3. Inhibition of BK1 cytotoxicity by Fas-Ig. 2C11xNP-redirectioned BK1 cytotoxicity was tested against NP-modified mFas/LS178Y in the presence of the indicated concentrations of normal human IgG (○) or Fas-Ig (●) in a 6-hr <sup>51</sup>Cr release assay. Data represent mean  $\pm$  SD of triplicate samples.

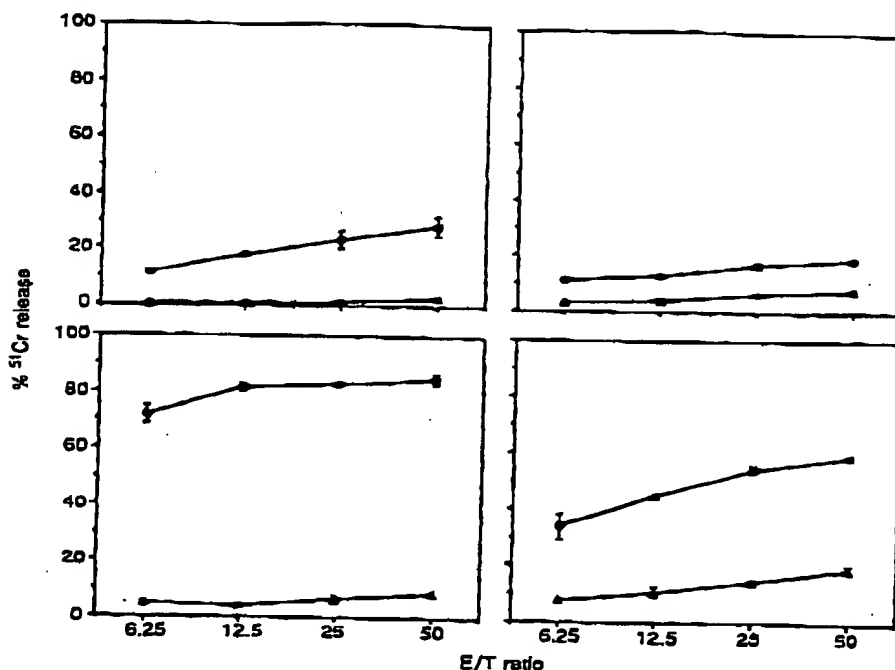


FIG. 4. Fas-mediated cytotoxicity exerted by splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Cytotoxic activity of splenic CD4<sup>+</sup> T cells (Left) and CD8<sup>+</sup> T cells (Right) was tested against NP-modified mFas/L5178Y (●) or mFasΔCyt/L5178Y (○) in the presence of 2C11αNP in a 6-hr (Upper) or 16-hr (Lower) <sup>51</sup>Cr release assay. Data represent mean ± SD of triplicate samples.

involvement of the Fas ligand in the BKL-mediated cytotoxicity.

**Fas-Mediated Cytotoxicity Exerted by Fresh CD4<sup>+</sup> and CD8<sup>+</sup> T Cells.** We next examined functional expression of the Fas ligand in freshly prepared T cells by utilizing the Fas-dependent target cell lysis system described above. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells purified from BALB/c spleen exerted substantial cytotoxicity against mFas/L5178Y as compared with that against mFasΔCyt/L5178Y after 16 hr of incubation (Fig. 4). The addition of Fas-Ig blocked the cytotoxicity (data not shown), indicating the involvement of Fas and its ligand. The Fas-mediated cytotoxicity was significantly detected even after 6 hr of incubation. Especially, CD4<sup>+</sup> T cells exerted strong cytotoxicity specifically against mFas/L5178Y, as compared with CD8<sup>+</sup> T cells, even at a low E/T ratio, suggesting that expression of the

Fas ligand might be inducible on the major population of these T cells.

**Inability of *gld* T Cells To Exert Fas-Mediated Cytotoxicity.** Mice carrying the *gld* mutation develop lymphadenopathy and systemic autoimmune disease and are of identical phenotypes to those developed in *lpr* mice (35). It has been suggested that the *lpr* and *gld* are mutations of genes encoding an interacting pair of molecules (36). The identification of *lpr* as the Fas defect (34) suggested that *gld* would result from the Fas ligand defect (37, 38). We addressed this possibility by testing the Fas-mediated cytotoxic activity of *gld* T cells. As indicated in Fig. 5, CD4<sup>+</sup> splenic T cells prepared from C3H *gld/gld* mice did not exert the Fas-mediated cytotoxicity, whereas those from C3H+/+ mice efficiently lysed the mFas/L5178Y. Although the genetic basis of the Fas ligand defect in *gld* mice remains to be

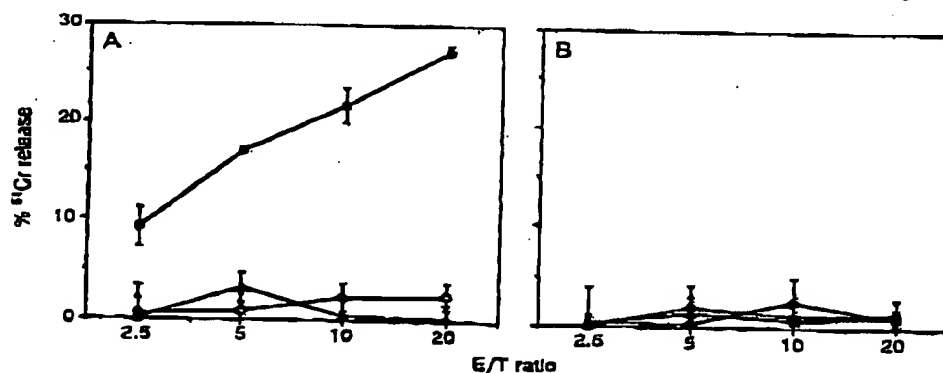


FIG. 5. Inability of *gld* T cells to exert Fas-mediated cytotoxicity. Cytotoxic activity of splenic CD4<sup>+</sup> T cells prepared from C3H+/+ (A) or C3H *gld/gld* (B) mice was tested against NP-modified L5178Y (○), mFas/L5178Y (●), or mFasΔCyt/L5178Y (△) in the presence of 2C11αNP in a 16-hr <sup>51</sup>Cr release assay. Data represent mean ± SD of triplicate samples.



determined, this result clearly indicates a defective expression of functional Fas ligand on *gld* T cells.

**Concluding Remarks.** In this study, we have demonstrated that the Fas/Fas ligand lytic pathway is a general one in T-cell-mediated cytotoxicity. Under normal and pathological conditions *in vivo*, the Fas-mediated cytotoxic pathway is limited by the expression of Fas on the target cells and also by the resistance of target cells to Fas-mediated apoptosis induction (39, 40). Recently, Fas expression has been demonstrated in various tissues (29), and it has been reported that the administration of an agonistic anti-Fas mAb into mice rapidly caused fulminant hepatitis by inducing drastic hepatocyte apoptosis (32). On the other hand, it has been well documented that the tissue injury accompanied with some organ-specific autoimmune diseases, such as insulin-dependent diabetes mellitus and Sjögren syndrome, as well as viral hepatitis is associated with massive T-cell infiltration (41–43). Our results suggest that these molecules would be very important targets of clinical intervention in such aggressive diseases.

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